Cyclic amphipathic peptide–DNA complexes mediate high-efficiency transfection of adherent mammalian cells

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ABSTRACT   A DNA transfection protocol has been developed that makes use of the cyclic cationic amphipathic peptide gramicidin S and dioleoyl phosphatidylethanolamine. The DNA complex is formed by mixing gramicidin S with DNA at a 1:1 charge ratio and then adding phosphatidylethanolamine at a lipid/peptide molar ratio of 5:1. The complex mediates rapid association of DNA with cells and leads to transient expression levels of β-galactosidase ranging from 1 to 30% of the transfected cells, with long-term expression being about an order of magnitude lower. The respective roles of peptide and phospholipid are not yet resolved but optimal transfection requires both the cyclic peptide and the hexagonal phase-competent phospholipid PtdEtn. Transfection in CV-1 cells is not affected by lysomotrophic agents, which suggests that DNA entry into the cell is via the plasma membrane. This technique that is simple, economical, and reproducible mediates transfection levels up to 20-fold higher than cationic liposomes in adherent mammalian cells.

Advances in gene therapy depend to a large degree on the development of delivery systems capable of efficiently introducing DNA into the target cell. Although significant progress has been made with retroviruses (1) or adenoviruses (2) for gene delivery, concern about possible recombination with endogenous virus, oncogenic effects, and immunologic host–response reactions has encouraged a search for nonviral DNA transfection techniques (3). Nonviral techniques, including cationic or pH-sensitive liposomes (4, 5), polylysine conjugates (6, 7), and direct injection of DNA (8) overcome some of the problems of the viral systems. However, there remains a need for improved transfection efficiency in the nonviral systems.

Since the efficiency of the viral systems is in large part due to fusogenic amino acid sequences on viral membrane proteins, we examined whether membrane-permeabilizing peptides could be used to create systems that efficiently transfect mammalian cells. The system developed here takes advantage of the DNA binding ability and the membrane destabilization properties of gramicidin S, an amphipathic cyclic decapeptide that bears two positive charges from ornithine residues on one side of the ring and the side chains of the hydrophobic residues arrayed on the opposite face of the ring (9). Gramicidin S, a membrane binding peptide (10), strongly interacts with nucleotides (11) and nucleic acids (12) by charge interactions and mediates phase transfer of these molecules into organic solvents. Gramicidin S also can destabilize membranes causing an increase in the permeability of liposomal (13), bacterial (14), and erythrocyte membranes (13).

We have created transfecting particles by complexing plasmid DNA with gramicidin S and then coating this complex with fusogenic lipids. In this paper, we describe the

optimization of the complex. We also show the ability of this system to transfect adherent mammalian cells in culture with a high efficiency for transient and stable expression of DNA.

MATERIAL AND METHODS

Expression Vectors. The plasmids pCluc4 (5 kb) (15) coding for firefly luciferase and pCMVβgal (7 kb) (16) coding for β-galactosidase were generous gifts from E. Wagner (Institute of Molecular Pathology, Vienna, Austria) and G. McGregor (Howard Hughes Medical Institute, Houston), respectively. Plasmid pSV2neo (Promega) has been described (17). Plasmids were grown and then purified on CsCl gradients (18). Plasmid pCluc4 was radiolabeled with ^{125}I-labeled dCTP or [^{32}P]dCTP by using a nick-translation kit (Bethesda Research Laboratories).

Complex Preparation. All lipids were purchased from Avanti Polar Lipids. Liposomes were prepared by drying 4 μmol of lipids under nitrogen at room temperature and by rehydrating the film with 4 ml of 30 mM Tris·HCl (pH 8.5). Liposomes were subsequently sonicated 15 min under argon by using a bath sonicator (Laboratory Supplies, Hicksville, NY). A typical complex preparation was made at room temperature by diluting 20 μg of plasmid DNA with 300 μl of 30 mM Tris·HCl (pH 8.5) in a polystyrene tube. Gramicidin S was diluted with 30 mM Tris·HCl (pH 8.5) to 2 mg/ml from a stock solution at 20 mg/ml in dimethyl sulfoxide. The diluted gramicidin S (20 μl/40 μg) was added to the DNA and quickly mixed. Then 175 μl of liposomes (equivalent to 175 nmol of lipids) was slowly added with gentle mixing to the DNA/gramicidin S mixture. The hydrodynamic diameter of the complex and the precursor liposomes was determined by dynamic light scattering (Malvern Instruments, Southborough, MA).

Complexes of DNA and lipids with other peptides, tyrocidine (United States Biochemical), polymyxin B (Sigma), melittin (Sigma, HPLC grade), and polylysine 100 (Sigma) were formed in the same way.

Cationic Lipid Reagent. The lipofection technique was used as a reference for all transfection studies. N-[1,(2,3-Dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA, 95% pure) was a gift from J. Senior (Syntex) and R. Debs (University of California, San Francisco). The lipofection reagent was prepared by mixing DOTMA and phosphatidylethanolamine (PtdEtn) in a 1:1 (wt/wt) ratio (4, 19). The diameter of the lipofection reagent–DNA complex was ≈520 nm, as measured by dynamic light scattering. An optimum DNA/lipofection reagent ratio of 1:10 (wt/wt) was used for transfection. This ratio corresponds to a lipofection reagent/DNA charge ratio of 2:1.

Characterization of the Peptide–DNA Complex by Physical Techniques. To estimate the interaction between the DNA

and the gramicidin S, plasmid DNA (1 μg) was mixed with gramicidin S (0–20 μg) and electrophoresed on a 0.7% agarose gel in the presence of ethidium bromide. The peptide–DNA–lipid complex was also analyzed by separation on a sucrose gradient. Since tyrocidine bears a tyrosine residue and was easier to label than gramicidin S, it was used to prepare the complexes that were analyzed on gradients. Tyrocidine was labeled with ^{125}I by the chloramine-T technique (20) and separated from free iodine on a Sephadex G-10 column. Purity of the final product was checked by HPLC. Complexes were made as described above with traces of ^{125}I-labeled tyrocidine, ^{32}P-labeled plasmid, and 1% rhodamine-conjugated PtdEtn. An aliquot (200 μl) of the complex (i.e., 8 μg of pCluc4 plasmid, 16 μg of tyrocidine, and 70 nmol of lipids) was layered on to a linear sucrose gradient [2–42% (wt/wt) sucrose in 30 mM Tris·HCl (pH 8.5)] and centrifuged at 190,000 × g at room temperature for 3 h. Gradients were fractionated and analyzed for each constituent by measuring radioactivity (for tyrocidine and DNA) or fluorescence (for PtdEtn). Transfection activity of each fraction was assayed on CV-1 cells.

Cells and Transfection Protocol. Cell lines CV-1 (monkey fibroblast), p388D1 (murine macrophage), RAW264.7 (murine macrophage), HeLa (human carcinoma cell), HepG2 (human hepatoma cell), and EL-4 (murine T cell) were provided by the University of California cell culture facility. Cells were cultured at 5 × 10⁵ cells per 60-mm culture dish and incubated in appropriate medium overnight at 37°C in 5% CO_2/95% air. In a typical experiment, cells were transfected in serum-free medium with 4 μg of plasmid per dish at 37°C, and 5 h later, medium was removed and replaced by medium containing 10% (vol/vol) fetal calf serum (FCS). In some experiments, medium containing 50% FCS was used during the first 5 h of transfection. Expression of luciferase (15) and β-galactosidase (16) genes was measured after 48 h of incubation at 37°C in 5% CO_2/95% air as described (19). The effect of lysomotrophic agents, chloroquine (100 μM), monensin (10 μM), and NH_4Cl (20 mM) on short-term expression was investigated by simultaneously adding these agents and the complex. Long-term expression experiments were done by cotransfection of CV-1 cells with 7 μg of pCMVβgal and 3 μg of pSV2neo plasmids per T-25 flask (25 cm²) of confluent cells. Twenty-four hours after transfection, cells were transferred to new T-25 flasks at 1 × 10⁴ cells per flask. Geneticin (G418, GIBCO) was added to 0.8 mg/ml and the cells were maintained under selective pressure for 3 weeks with a change of medium every week.

Cell-Associated DNA. The complexed plasmid pCluc4 (4 μg) containing traces of ^{125}I-radiolabeled plasmid was added to CV-1 cells as described in the transfection protocol. After 10 min, 30 min, 2 h, and 5 h, medium was removed and cells were rinsed five times with ice-cold calcium- and magnesium-free phosphate-buffered saline (0.2 g of KH_2PO_4, 2.16 g of Na_2HPO_4, 0.2 g of KCl, and 8.0 g of NaCl per liter). Cells were solubilized in 1 ml of 0.5 M NaOH and cell lysates were assayed for radioactivity in a γ counter (Gamma 8000, Beckman) and for proteins by the method of Bradford (Bio-Rad kit).

Toxicity Assay. Toxicity of the peptide complex was compared to that of cationic liposomes on p388D1 and CV-1 cells by using a colorimetric dye reduction assay (21). Cells were plated at 5 × 10⁴ cells per well in a 96-well tray and 0.1–2 μg of pCluc4 plasmid complexed with peptide–PtdEtn or cationic liposomes [DNA/lipofection reagent, 1:10 (wt/wt)] was added per well in 100 μl of serum-free medium. After a 5-h incubation, medium was removed and replaced by 100 μl of medium containing 10% FCS. Cells were incubated at 37°C in 5% CO_2/95% air for 24 h. Results are expressed as the percentage of dye reduction in cells treated with the trans-

fection system compared to dye reduction in cells treated with buffer.

RESULTS

Formation of the Peptide–DNA Complex. The DNA/gramicidin S ratio for maximal transfection was determined at a PtdEtn/gramicidin S constant molar ratio of 4.5:1. Maximal transfection occurred when the ratio between gramicidin S and DNA corresponded to a 1:1 charge ratio (Fig. 1A). The ratio of lipid to gramicidin S was also important and maximal transfection occurred at a PtdEtn/gramicidin S ratio
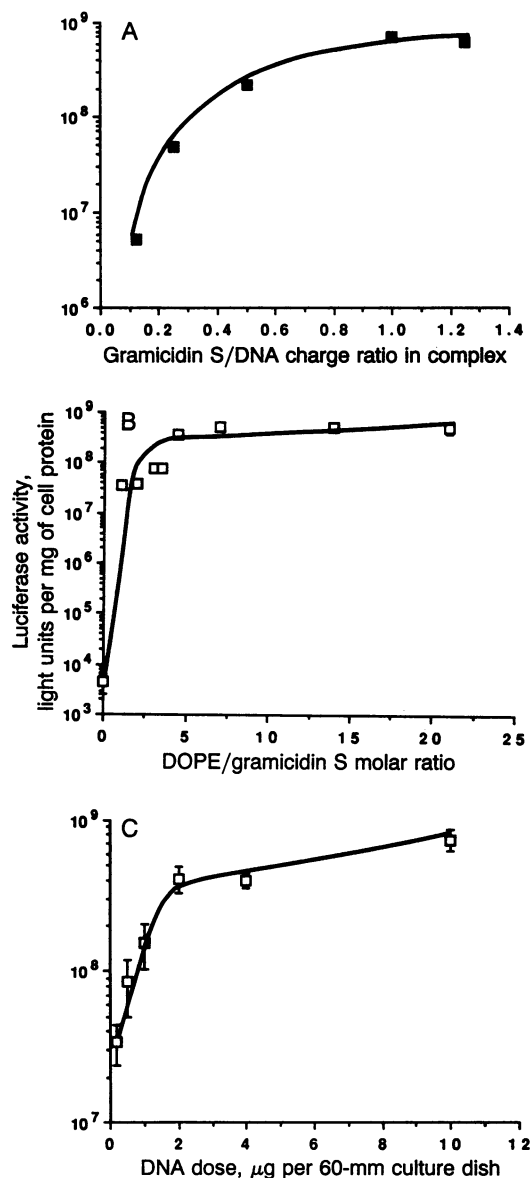

FIG. 1. Influence of transfection complex components and concentrations on expression of luciferase reporter gene in CV-1 cells. (A) Effect of gramicidin S/DNA charge ratio on transfection. The various complexes were prepared with various gramicidin S/DNA charge ratios at a PtdEtn/gramicidin S molar ratio of 4.5:1. Each point is the mean of duplicate transfections. (B) Effect of the PtdEtn/gramicidin S molar ratio on transfection. Complexes were prepared with various PtdEtn/gramicidin S molar ratios at a gramicidin S/DNA charge ratio of 1. Each point is the mean ± SD of triplicate transfections. DOPE, dioleoyl PtdEtn. (C) Effect of DNA dose on transfection. The optimized complex (gramicidin S/DNA charge ratio = 1; PtdEtn/gramicidin S molar ratio = 5) was incubated with CV-1 cells at various concentrations of pCluc4 plasmid per dish for 5 h. Each point is the mean ± SD of triplicate transfections.

of between 4 and 5 (Fig. 1*B*). In the absence of lipids, a low level of transfection was observed. At very high PtdEtn/gramicidin S ratio (>25), the complex precipitated and transfection efficiency was reduced (data not shown).

Thus, maximal transfection took place with the addition of 4 μg of DNA in 2 ml of medium, corresponding to 3.5 μM gramicidin S and 17.5 μM PtdEtn. The optimal complex had a diameter of 330 $\pm$ 50 nm, whereas the precursor liposomes had a diameter of 120 $\pm$ 40 nm (mean $\pm$ SD of three determinations).

Transfection efficiency in CV-1 cells showed a linear increase as the amount of DNA added increased from 0.2 to 2 μg (Fig. 1*C*). Luciferase expression reached a plateau at this amount of DNA, which represents $\approx 10^6$ copies of plasmid per cell. The time of incubation of the optimized complex with the cells had only a small effect on the final level of luciferase expression. Forty-eight hours after a 10-min incubation of the complex with cells, luciferase activity was $>10^7$ light units per mg of cell protein, suggesting a rapid cell–complex interaction. Incubation of the complexes with the cells for >5 h neither increased luciferase expression nor resulted in any visible toxicity to CV-1 cells (data not shown).

We used agarose gel electrophoresis and sucrose sedimentation gradients to gain some insight into the nature of the interactions between the components in the complex. Plasmid DNA was not completely retarded in agarose gel electrophoresis until the gramicidin S/DNA charge ratio was 5:1 (Fig. 2*A*). However, when PtdEtn was added to the gramicidin S/DNA mixture, gel retardation of the complex occurred at a gramicidin S/DNA charge ratio of 1:1 (data not shown). Analysis of the sucrose gradients of the complex indicated that two major peaks of DNA could be separated (Fig. 2*B*). The first peak contained almost all the PtdEtn and led to low transfection levels. The second peak corresponded to more dense material and allowed two to three orders of magnitude higher transfection of CV-1 cells than the lipid-rich fraction. In comparison, DNA alone migrated at the middle of the gradient and DNA complexed with tyrocidine migrated at the bottom of the gradient. The lipofection reagent–DNA complex remained at the top of the gradient (data not shown) as reported (4).

Role of Peptide and Lipid Components in the Transfection Complex. To determine whether charge neutralization or the amphipathic nature of the peptide was more important in the action of the peptide component, we studied the ability of other cationic peptides to form a transfecting complex with DNA and PtdEtn (Fig. 3*A*). Tyrocidine, a gramicidin S analog that contains only one positive charge, was able to replace gramicidin S in the complex when used at a 1:1 charge ratio with DNA. When the positive charge of gramicidin S was eliminated by covalent modification of the ornithine, transfection activity was lost (data not shown). Polymyxin B, a cationic cyclic peptide, formed a transfecting complex at a 1:1 charge ratio that was five orders of magnitude less efficient. Polylysine (10 kDa) could also form a transfecting complex with PtdEtn and DNA at a 1:1 charge ratio that was 1000-fold less active than that formed by gramicidin S. When the charge on the DNA was neutralized by a mixture of polylysine and gramicidin S, the transfection did not decrease as long as 25% of the positive charges came from gramicidin S. Since transfection decreased 1000-fold in the absence of gramicidin S, this suggests that the hydrophobic face of gramicidin S is important for transfection activity.

Finally, the linear cationic peptide melittin was able to form a transfecting complex that was four orders of magnitude less effective than gramicidin S. In this case, the 1:1 complex was too cytotoxic to be applied to the cells and the complex was prepared at a peptide/DNA charge ratio of 1:8.
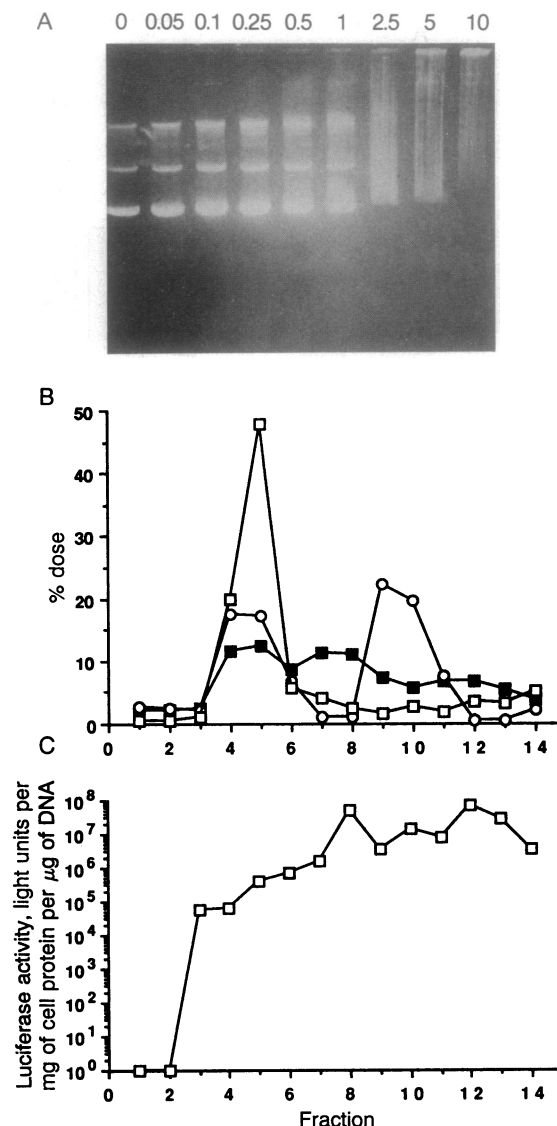

FIG. 2. Characterization of the peptide–DNA complex by physical techniques. (*A*) Agarose gel electrophoresis of DNA–gramicidin S complexes. Gramicidin S/DNA charge ratio is indicated above each lane. (*B*) Separation of the tyrocidine–DNA–PtdEtn complex on sucrose sedimentation gradient. Separation profiles of DNA (○), tyrocidine (■), and PtdEtn (□) are indicated as the percent of dose applied to the gradient. (*C*) Transfection efficiency of each fraction is expressed as luciferase activity per 60-mm dish of CV-1 cells.

We used other phospholipids to form the peptide complex to examine the hypothesis that the ability of the phospholipids to enter the hexagonal phase significantly enhances the transfecting competence of the lipids (Fig. 3*B*). Addition of methyl moieties to the PtdEtn head group [monomethyl PtdEtn, dimethyl PtdEtn, and phosphatidylcholine (PtdCho)], which decreases the propensity of the phospholipid to form the hexagonal phase, decreased transfection. Transfection levels also decreased when phospholipids with higher phase transition temperatures than PtdEtn, such as palmitoyloleoyl PtdEtn or dipalmitoyl PtdEtn, were used. PtdEtn/PtdCho at a 2:1 molar ratio and PtdEtn alone mediated expression of luciferase similarly. When dimyristoyl PtdEtn, dilauroyl PtdEtn, or diphytanoyl PtdEtn replaced PtdEtn in the complex, no transfection was observed.

Transfection of Mammalian Cells with Peptide–Lipid–DNA Complex. Gramicidin S-mediated transfection was 2- to 20-fold higher than cationic liposomes in CV-1, HeLa, HepG2, and RAW 264.7 cells (Table 1). However, cationic liposomes
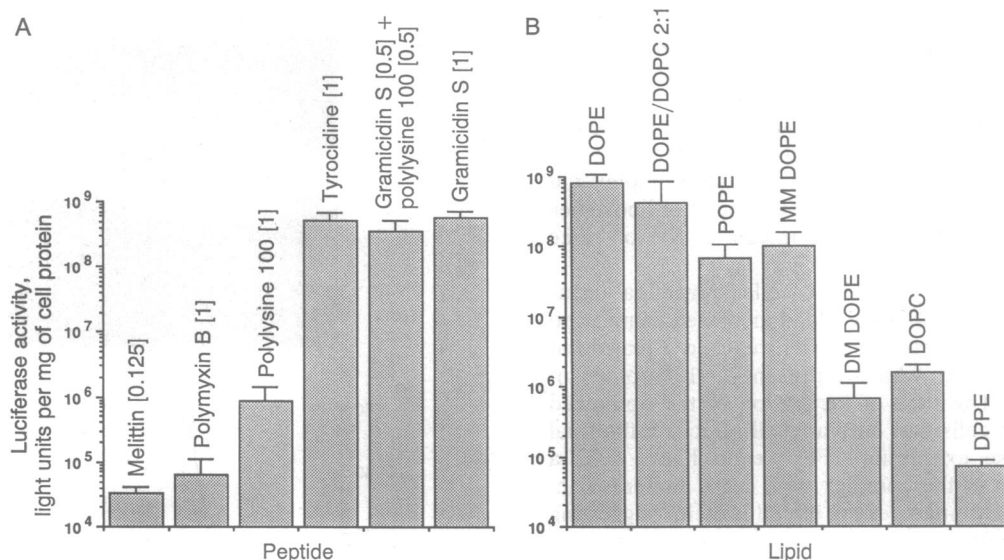
FIG. 3. Influence of peptide or phospholipid components on expression of luciferase reporter gene in CV-1 cells. (A) Effect of peptide on transfection. Various complexes were prepared with different peptides, gramicidin S, tyrocidine, polymyxin B, polylysine, and melittin. The amount of PtdEtn in the complexes was 175 nmol (lipid/peptide molar ratio was 5:1). The peptide/DNA charge ratio is indicated in brackets. (B) Effect of lipid on transfection. Complexes were prepared with various phospholipids and a lipid/gramicidin S molar ratio of 5:1. The gramicidin S/DNA charge ratio was constant at 1:1. Each point is the mean ± SD of triplicate transfections. DOPE, dioleoyl PtdEtn; DOPC, dioleoyl PtdCho; POPE, palmitoyloleoyl PtdEtn; DPPE, dipalmitoyl PtdEtn; MM, monomethyl; DM, dimethyl.

mediated higher transfection in p388D1 and EL-4 cells. The reason why the peptide complex could not transfect suspension cell line EL-4 is still unclear. However, in contrast to lipofection, the gramicidin S complex did not agregate EL-4 cells. This observation suggests that aggregation of the cells mediated by the cationic liposomes may facilitate entry of DNA into suspension cells. For CV-1 cells, the gramicidin S–lipid–DNA complex was also more efficient than cationic liposomes for the transfection of plasmids encoding β-galactosidase. Twenty to 30% of the cells treated with the peptide complex expressed β-galactosidase activity whereas cationic liposomes induced 2- to 5-fold fewer β-galactosidase-positive cells (data not shown). When the β-galactosidase was co-transfected with a plasmid coding for the enzyme conferring neomycin resistance, expression of β-galactosidase 3 weeks after transfection ranged from 1 to 5% of the cells initially exposed to the plasmids (data not shown). Medium containing 50% FCS had an inhibitory effect on the transfection level in CV-1 cells by using both the lipofection reagent (4, 19) and the peptide complex. Luciferase activity decreased ≈50-fold compared to transfection in serum-free medium.

Uptake of Plasmid DNA by CV-1 Cells. Cell-associated delivered DNA was measured (Fig. 4). Only 1–2% of uncomplexed DNA was cell-associated at the end of a 5-h incubation. The peptide–lipid–DNA complex rapidly associated with the cell but the cell-associated DNA did not increase with time. The final level of cell-associated DNA induced by the cationic

Table 1. Transfection of various mammalian cell lines with peptide complex or cationic liposomes

Cell line	Luciferase activity	
	Cationic liposomes	Peptide complex
CV-1	$1.2 \pm 0.7 \times 10^8$	$6.8 \pm 1.9 \times 10^8$
HeLa	$5.8 \pm 1.3 \times 10^6$	$3.3 \pm 0.6 \times 10^7$
HepG2	$1.3 \pm 0.1 \times 10^7$	$2.0 \pm 0.3 \times 10^8$
p388D1	$4.8 \pm 0.9 \times 10^6$	$1.4 \pm 0.4 \times 10^6$
RAW264.7	$3.5 \pm 1.3 \times 10^4$	$8.9 \pm 6.4 \times 10^4$
EL-4	$1.1 \pm 0.06 \times 10^5$	0

Results are expressed as light units per mg of cell protein and are the mean ± SD of triplicate dishes in the same experiment.

liposomes (40–50% of the applied dose per mg of cell protein) was ≈5-fold greater than that induced by the peptide complex (6–10% of the applied dose per mg of cell protein).

Addition of lysomotrophic agents, chloroquine, monensin, and ammonium chloride, at the beginning of the 5-h incubation did not significantly change the transfection level induced by the peptide complex in CV-1 cells. These agents have been shown to increase expression of luciferase after plasmid delivery via cationic liposomes in CV-1 cells (19).

Comparison of Cytotoxicity of Cationic Liposomes to the Peptide Complex. In the CV-1 cell line, the overall toxicity of both cationic liposomes and the peptide complex was low. When PtdEtn was omitted in the complex, gramicidin S–DNA complexes lowered dye reduction to 30% of control values.

DISCUSSION

The rationale for using the cyclic peptide gramicidin S for plasmid DNA delivery was based upon two interesting prop-
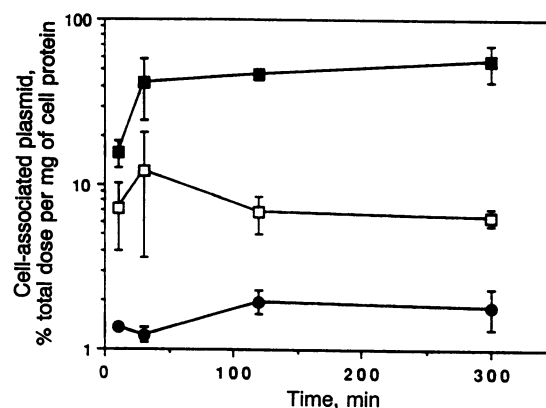

FIG. 4. Kinetics of cell association of plasmid DNA when delivered by the peptide complex or the cationic liposomes. CV-1 cells were incubated with pCluc4 plasmid alone (●) or pCluc4 plasmid associated with the cationic liposomes (■) or the peptide complex (□). Plasmid (4 μg) was added per 60-mm dish. Each point is the mean ± SD of triplicate dishes.

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erties: (*i*) gramicidin S strongly binds DNA by charge interactions and (*ii*) gramicidin S is an amphipathic peptide that can permeabilize cell membranes. We discovered that gramicidin S can facilitate DNA delivery into mammalian cells. However, to obtain high transfection levels, phospholipids are also required in the complex with DNA. The phospholipids appear to have two functions: (*i*) to decrease the cytotoxicity of the gramicidin S and (*ii*) to enhance the transfection level. The ability of a phospholipid to enhance transfection is correlated with its ability to undergo a lamellar–hexagonal transition (PtdEtn > palmitoyloleoyl PtdEtn > monomethyl PtdEtn). Phospholipids that cannot undergo this transition at temperatures <75°C (dilauroyl PtdEtn, dimyristoyl PtdEtn, dipalmitoyl PtdEtn, diphytanoyl PtdEtn, and PtdCho) were orders of magnitude less effective than PtdEtn. Clearly, there are other factors involved and the correlation does not take into account any change in the lipid-phase behavior that the other components in the complex induce.

As far as the peptide component is concerned, both a positive charge and an amphipathic character are important for high transfection. In addition, only the related cyclic peptide tyrocidine mediated a similar level of gene transfer at the same DNA/peptide charge ratio. Interestingly, this optimal ratio corresponds to a 1:1 charge ratio between the plasmid DNA and the peptide. However, a 1:1 charge ratio, even if required, is not sufficient to lead to efficient transfection since polylysine or polymyxin B, a cyclic peptide, at a 1:1 charge ratio with DNA did not mediate high transfection. The melittin-based complex could not lead to efficient transfection even though this peptide, like gramicidin S, can also disrupt membranes (22). For melittin, its apparent partition coefficient for membranes is about two orders of magnitude lower than that of gramicidin S. Moreover, melittin forms an α-helical-like structure in membranes, whereas gramicidin S exists in a β-sheet conformation. The high affinity for membranes as well as the β-sheet conformation may be important for formation of the specific structural interaction of the DNA, lipid, and peptide that creates a high-efficiency transfecting complex.

We believe the mechanism of DNA delivery by the peptide–lipid–DNA complex involves entry of the DNA through the plasma membrane. This supposition is based upon the fact that PtdEtn can enhance membrane fusion (23) and that gramicidin S can permeabilize cell membranes. Consistent with this proposal are the findings in CV-1 cells that the cell association of DNA is rapid, transfection is high (even after a short incubation of the complex with the cells), and lysomotrophic agents do not influence the extent of transfection. These characteristics distinguish the peptide-mediated DNA delivery from that induced by pH-sensitive liposomes (19), which follow an endosomal pathway.

For pH-sensitive liposomes, which deliver their contents via the endosomal/lysosomal route (24), there is a gradual increase in transfection as the incubation time increases and transfection is significantly reduced by lysomotrophic agents (19, 24). For cationic liposomes, which appear to have multiple pathways for DNA delivery, there is a high level of cell association that increases with incubation time and transfection is significantly increased by lysomotrophic agents in CV-1 cells (19). In CV-1 cells, the peptide complex brings about less DNA–cell association than the cationic liposomes yet causes more transfection. Perhaps increasing the level of cell association of the peptide complex by targeting to cell surface components would lead to greater transfection levels with the peptide complex, as has been

found with pH-sensitive liposomes (25, 26) and the polylysine complexes (6, 7).

The gramicidin S–PtdEtn–DNA complex described here is a simple and economical reagent for high levels of transfection. Moreover, the observation that amphipathic peptides in conjunction with phospholipids can significantly increase DNA transfection opens up an avenue for the creation of DNA delivery complexes with even higher transfection efficiencies than currently available protocols.

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